Cloning and Characterization of Fetal Liver Phosphatase 1, a Nuclear Protein Tyrosine Phosphatase Isolated From Hematopoietic Stem Cells

By Mercedes Dosil, Neville Leibman, and Ihor R. Lemischka

We report the isolation of cDNAs encoding protein tyrosine phosphatases (PTPs) from highly purified hematopoietic stem cell populations. One such cDNA encodes a novel PTP, designated fetal liver phosphatase 1 (FLP1), which consists of one PTP domain followed by a carboxy terminal domain of 160 amino acids. Northern blot and in situ hybridization analysis showed that expression of FLP1 mRNA is restricted to thymus in 15.5-day-old and 17.5-day-old mouse embryos and to kidney and hematopoietic tissues in adult mice. Furthermore, polymerase chain reaction-based analysis shows that FLP1 is expressed in hematopoietic stem cells as well as in more mature hematopoietic cells. Peptide antisera against FLP1 immunoprecipitated a 48-kD protein that is localized in the nuclei of Ba/F3 lymphoid cells. We have analyzed the effects of overexpressing either wild-type FLP1 or a functionally inactive mutant of FLP1 in hematopoietic cells. In the progenitor K562 cell line, cells ectopically expressing functional FLP1 differentiated normally to megakaryocytes after induction with tetradecanoyl phorbol acetate (TPA). In contrast, when K562 transfectants expressing an inactive mutant FLP1 protein were treated with TPA, the characteristic cell spreading and substrate adhesion that accompany megakaryocytic differentiation did not occur. We show that, in these cells, the induction of the differentiation marker $\alpha_{IIb}\beta_3$ is not affected. However, both constitutive and TPA-induced expression of α_2 integrin, a late megakaryocytic marker, are inhibited. These results suggest that the expression of an inactive form of FLP1 affects late signaling events of K562 megakaryocytic differentiation.

THE HEMATOPOIETIC system continuously produces at least eight different blood lineages.[1,2] At the center of this system is a population of hematopoietic stem cells that can both self-renew and produce committed progenitors for all blood cell lineages. Traditionally, hematopoietic stem cells have been studied as activities either by transplantation contexts or in complex in vitro culture systems. Recently, efforts in a number of laboratories have yielded procedures for the purification of stem cells from adult bone marrow and fetal liver.[3-6] Thus, it has become possible directly to analyze the mechanisms that control stem cell behavior and identify the molecules that regulate self-renewal and commitment decisions.

Hematopoietic cells are regulated in part by polypeptide factors and their cognate receptors. Although several hematopoietic receptors are protein tyrosine kinases, the majority of hematopoietic growth factor receptors belong to a large cytokine receptor superfamily that does not have intrinsic kinase activity. Nevertheless, ligand binding to cytokine receptors generally leads to tyrosine phosphorylation of intracellular proteins.[7,8] These phosphorylation events are regulated by the coordinated activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs).

Both PTKs and PTPs are encompassed by large and diverse families of transmembrane and intracellular molecules. In the case of the PTP family,[9-11] each member contains at least one catalytic domain of approximately 250 amino acids that is characterized by the conserved signature motif (I/V)HCXAGXGR(S/T)G. Within this sequence there is an invariant cysteine residue essential for catalytic activity. The overall structure of receptor-like PTPs contains an intracellular segment with one or two phosphatase domains, a single transmembrane domain, and a variable extracellular segment with putative ligand binding activity. Intracellular PTPs possess a single catalytic domain flanked at either the amino or carboxy terminus by unrelated sequences of variable length. Some of these noncatalytic sequences include Src homology 2 (SH2) domains; segments of homology to the cytoskeletal protein band 4.1 or to lipid binding proteins; motifs that direct association with intracellular membranes; and sequences rich in proline, serine, threonine, and acidic residues (PEST sequences).[9-11]

Despite the large number of PTPs identified, relatively little is known about their biologic roles and mechanisms of action. Two of the best studied PTPs, the cytoplasmic SHP1 (also called HCP, PTP1C, or SH-PTP1) and the receptor-like CD45, are predominantly expressed in hematopoietic tissues and are known to play pivotal roles in hematopoiesis.[12-14] Naturally occurring mutations in the SHP1 gene are the cause of the severe hematopoietic disorder in *motheaten* mice[12-14] and gene-targeted mutations in CD45 block T-cell maturation.[15]

Because PTPs reverse the action of PTKs, including growth factor receptors and oncogene products that stimulate proliferation, it may be predicted that they transmit growth-inhibitory signals. Consistent with this idea is the function of SHP1, which is known to downmodulate signals mediated by interleukin-3 (IL-3),[16] erythropoietin,[17] interferon,[18] B-cell antigen receptor,[19] and CD22.[20] Alternatively, PTPs may induce growth. This has been shown in the case of CD45, which is a positive regulator in antigen-stimulated proliferation of T lymphocytes.[13] Other PTPs are known to have roles in processes other than cell proliferation. Examples of these are PTPκ and PTPμ, two receptor-like proteins implicated in regulating cell adhesion,[21-23] and PTP-BAS, an intracellular PTP that inhibits apoptotic signals mediated by the FAS

From the Department of Molecular Biology, Princeton University, Princeton, NJ.

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Address reprint requests to Ihor R. Lemischka, PhD, Department of Molecular Biology, Princeton University, Washington Road, Princeton, NJ 08544-1014.

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receptor.[24] For many PTPs that have unique structural features or that, like the PEST-containing PTPs, constitute subfamilies with no identifiable functional motifs, the biologic functions still remain largely unknown.

Several PTPs have already been identified in hematopoietic cells.[25-27] However, the functional roles of these enzymes in stem cell biology are unclear. We report the identification of PTPs expressed in highly purified stem cell populations using a polymerase chain reaction (PCR) cloning approach. Thirteen distinct PTP sequences were identified. Expression patterns in hematopoietic stem cells, pluripotent progenitors, and mature hematopoietic populations have been analyzed. One novel PTP, designated fetal liver phosphatase 1 (FLP1), has been characterized. FLP1 is a nuclear protein with structural similarities to PEST-containing PTPs. It is primarily expressed in primitive and more mature hematopoietic cells. We have analyzed the effects of overexpressing either wild-type FLP1 or a functionally inactive mutant of FLP1 in several hematopoietic cell lines. Deregulated expression of a catalytically inactive mutant of FLP1 in hematopoietic progenitor K562 cells inhibits cell spreading and substrate adhesion during in vitro megakaryocytic differentiation. One mechanism for this effect might be a downregulation of α_2 integrin expression and inhibition of α_2 integrin induction during the differentiation process.

MATERIALS AND METHODS

Cell purification. C57BL/6 mice were used in all experiments. Stem cell purifications have been described previously.[3,28,29] In brief, midgestation (day-14) fetal liver cells were fractionated into AA4.1 positive by immunopanning. The cells were stained with fluorescein isothiocyanate (FITC)-labeled lineage (Lin) cocktail (anti-Gr-1, Mac-1, B220, CD3, CD4, CD5, CD8, and TER-119), phycoerythrin (PE)-labeled anti-Ly6A/E (Ly6A), and biotinylated anti-c-Kit receptor followed by streptavidin-APC (Molecular Probes, Eugene, OR). For bone marrow, mononuclear cells were first fractionated by a Ficoll density gradient (<1.007), and lineage negative or low staining cells ($Lin^{-/lo}$) were obtained by magnetic separation with antirat IgG-coated magnetic beads (Dynalab, Rochester, NY) after staining with an FITC-Lin cocktail. The cells were further stained with PE-anti-Ly6A and biotinylated anti-c-Kit. Yolk sac cells from day-9 fetuses were separated by discontinuous gradient of Percoll (<1.077). Nonadherent cells were immunopanned with AA4.1 antibody and stained with FITC-labeled wheat germ hemaglutinin (FITC-WGA; Sigma, St Louis, MO). For purifications three-color analysis and sorting were performed using an Epics 753 dye laser (Coulter, Hialeah, FL) flow cytometer. All antibodies were purchased from Pharmingen (San Diego, CA) except TER-119 (a gift of Dr T. Kina, Kyoto University, Kyoto, Japan).

Identification of PTP cDNAs from fetal liver hematopoietic stem cells and PCR expression analysis. Preparation of total RNA from purified $AA4.1^+$, $Lin^{-/lo}$, $c\text{-}Kit^+$, Sca^+ fetal liver cells was performed as described.[30] Synthesis of cDNA was performed using SuperScript II RNase H-reverse transcriptase (GIBCO BRL, Grand Island, NY) and oligo(dT) primer. Purified cDNA was amplified with A and B degenerate PTP primers as described by Matthews et al.[25] The cycling parameters were 94°C for 30 seconds, 37°C for 30 seconds, and 72°C for 1 minute. In general, 60 cycles of amplification with the addition of primers and other components at midpoint were performed. Amplified cDNAs with sizes around 300 bp were subcloned into pSport1 (GIBCO BRL) through *Kpn* I and *Xba* I sites incorporated into the oligonucleotides. Bacterial transformants were screened for the presence of the appropriately sized inserts and random clones were chosen and sequenced. Sequences were compared with the EMBO/GenBank data base. Individual clones corresponding to PTPs were pooled and used as probes for sequentially screening a total of 1,300 colonies. For PCR expression analysis, total RNA from fetal liver, yolk sac, and bone marrow cell subpopulations was used for cDNA synthesis using oligo(dT) primer and PCR amplification with degenerate PTP primers. Cycling parameters were 1 minute at 94°C, 2 minutes at 42°C, and 3 minutes at 72°C for 48 cycles. Ten percent of the reaction mixtures were electrophoresed on 1.5% agarose gels. Southern blotted, and hybridized to ^{32}P-labeled DNA probes corresponding to the different PTP cDNAs isolated from fetal liver stem cells. Amplified cDNAs were normalized and controls for contamination were included in all amplifications. Results were always verified using independently synthesized cDNA templates. The expression patterns for FLP1, PTPβ, and DEP-1 were corroborated by Southern blot on DNAs from cDNA libraries of different hematopoietic populations.

Library construction and characterization of FLP1 cDNA. Fetal liver $AA4.1^+$ cells were obtained from a large number of day-14 mouse fetuses. RNA from these cells was purified by the guanidinium-thiocyanate-CsCl gradient method and poly(A)$^+$ RNA obtained by standard column protocols. For cDNA synthesis and cloning into the plasmid pSport1, we used a SuperScript Plasmid System kit (GIBCO BRL). The resulting directional library had an average insert size of 1 to 2 kb and an initial complexity of 5×10^6 clones. Two million recombinant clones of this library were screened with the 250-bp PCR fragment encompassing part of the FLP1 catalytic domain. Two cDNA clones encoding for amino acid positions 98 to 452 and extending to the 3' end of the mRNA were isolated. Antisense oligonucleotides corresponding to nucleotide positions 414 to 440 and 504 to 528 were used as gene-specific primers to extend the cloned cDNA in the 5' direction by using the 5'-Aplifinder RACE Kit (Clontech, Palo Alto, CA). The template for the amplifications was 5'-RACE-Ready kidney cDNA (Clontech). Independently amplified cDNA fragments were sequenced and two contiguous sequences of 1,560 bp (FLP1A) and 1,478 bp (FLP1B) were obtained. The authenticity of these cDNA sequences was further confirmed by PCR with primers corresponding to nucleotide positions 10 to 36 and 1515 to 1540, using as templates cDNAs from kidney and fetal liver $AA4.1^+$ cells. All DNAs were sequenced on both strands. The FLP1 cDNA sequence is under GenBank accession number U52523. The program PEST-FIND used for the search of PEST sequences was a kind gift of Dr M. Rechsteiner (University of Utah, Salt Lake City, UT).

Northern blot analysis and in situ hybridization. Twenty micrograms of total RNAs, isolated from a range of adult mouse tissues or cells lines by the method of Chomczynski and Sacchi,[31] were separated on formaldehyde-agarose gels and transferred to nylon membranes. Hybridization and washing conditions were as described.[32] The probe fragment encompassed amino acid positions 98 to 235. For comparison, filters were striped and rehybridized with a β-actin probe. For in situ hybridization studies, sense and antisense ^{35}P-labeled probes were synthesized by standard protocols from a pBlueScript (Stratagene, La Jolla, CA) plasmid containing a FLP1 cDNA fragment (nucleotides 1217 to 1373). As a positive control, we used the pKLS4 plasmid, which contains a cDNA fragment of the mouse Grb2 protein (kindly provided by K.L. Suen, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). In situ hybridizations were performed essentially as previously described by Wilkinson and Green.[33] Briefly, tissues were cryostat sectioned at 10 μm, mounted onto polycationic slides, and refixed in 4% paraformaldehyde in phosphate-buffered saline solution. Hy-

bridization was performed a 52°C in the presence of 2.5 to 4 × 10^6 cpm of ^{33}P-labeled riboprobe per milliliter. Posthybridization washings included treatment with 20 μg of RNase A (Boehringer Mannheim, Indianapolis, IN) at 37°C for 30 minutes and two high-stringency washings at 65°C with 50% formamide (Sigma), 2× SSC (1× SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate), and 100 mmol/L dithiothreitol (Boehringer Mannheim). After dehydration, slides were dipped into a solution containing 50% NTB-2 emulsion (Eastman Kodak, Rochester, NY) and 1% glycerol, exposed at 4°C for 15 to 20 days, and developed.

Antibodies and protein analysis. The α-tubulin antibody was obtained from Oncogene Science (Uniondale, NY) and the α_2 integrin antibody used for immunoblots was from Chemicon International Inc (Temecula, CA). The FLP1 antibody EN12 was generated by immunizing rabbits with a bacterially expressed glutathione S-transferase (GST) fusion protein containing residues 182 to 405 of the FLP1 protein. Two other polyclonal antibodies, BN1 and R93, against a bacterially expressed GST-fusion protein and a synthetic peptide encompassing FLP1 amino acids 277 to 441 and 327 to 345, respectively, were generated. The specificity of these antibodies was determined by immunoprecipitation and Western blot on COS cells transfected with pSV-Sport plasmids (GIBCO BRL) encoding hemagluinin-tagged versions of the FLP1A and FLP1B isoforms. Their efficiencies were analyzed by comparison to the efficiency of the antihemaglutinin antibody (Boehringer Mannheim). All three antibodies immunoprecipitated FLP1A and FLP1B proteins expressed in COS cells and cross-reacted with a 45/48-kD endogenous protein in different hematopoietic cell lines. Antibody EN12 was found to be more efficient in immunoprecipitation and immunoblotting of endogenous FLP1 proteins. Nuclear and cytoplasmic extracts were prepared using the method of Dignam et al,[34] and immunoprecipitations were performed as previously described.[33] The resulting immunecomplexes were bound to protein A-Sepharose beads (Pharmacia, Piscataway, NJ) and washed three times with buffer A (10 mmol/L Tris-Cl [pH 8.0], 150 mmol/L NaCl, 2 mmol/L EDTA, 0.2% Triton X-100), once with buffer B (10 mmol/L Tris-Cl [pH 8.0], 500 mmol/L NaCl, 2 mmol/L EDTA, 0.2% Triton X-100), and once with buffer C (10 mmol/L Tris-Cl [pH 8.0]). The immunoprecipitates were dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and fractionated in 8% acrylamide gels. Western blotting was performed by transferring proteins onto nitrocellulose membranes using a Milliblot-SDE transfer system (Millipore, Bedford, MA). Membranes were incubated with the appropriate antibody in 5% bovine serum albumin, Tris-Cl (pH 8.0), 0.9% NaCl. When polyclonal antibodies were used, immunoblots were subsequently incubated with ^{125}I-protein A and autoradiographed with intensifying screens at −70°C. Blots incubated with anti-α-tubulin and anti-α_2 integrin monoclonal antibodies were developed using the enhanced chemiluminescence Western blotting (immunoblotting) system (Amersham, Arlington Heights, IL).

Cell culture and DNA transfections. K562 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum plus antibiotics. The murine IL-3–dependent cell line Ba/F3 was maintained in RPMI 1640 supplemented with 10% fetal calf serum and 10% Wehi-3B conditioned medium. The expression plasmids contained the intact FLP1A or mutant FLP1B-CS cDNA sequences cloned in the pBabe retroviral expression vector.[36] The single amino acid mutation (C to S) in the FLP1A and FLP1B cDNAs was generated using the Altered Sites II in vitro mutagenesis kit (Promega, Madison, WI). K562 and Ba/F3 cells were transfected by electroporation. In brief, 2 × 10^6 cells were resuspended in 0.4 mL of culturing media and 20 μg of pBabe-FLP1A, pBabe-FLP1A-CS, or pBabe-FLP1B-CS linearized by digestion with *Sca* I and 1 μg of *Hind*III-digested pGKneo were added to the cell suspension. Cells were exposed to a 260-V pulse with a capacitance of 960 μF using a Gene Pulser electroporator (Bio-Rad, Melville, NY). After a growing period of 24 hours in their optimal medium, transfected cells were transferred to microtiter plates and selected in G418 (0.8 mg/mL; GIBCO). After 2 weeks, the cells were expanded as independent pools. Single-cell clones were obtained by limiting dilution and analyzed for protein expression.

Determination of PTP activity. Enzymatic activity of FLP1 immunoprecipitates from K562 clones overexpressing FLP1A, FLP1A-CS, and FLP1B-CS was determined using a malachite green microtiter-plate assay.[37] Cellular lysates from 50 × 10^6 cells were incubated with EN12 antibody and immunoprecipitates were collected and washed as described above. Enzyme reactions were performed in 96-well plates in a final volume of 25 μL. Substrates included tyrosine-phosphorylated peptides TSTEPQpYQPGENL and RRLIEDAEp-YAARG (Upstate Biotechnology, Lake Placid, NY), corresponding to the C-terminal regulatory and autophosphorylation sites of c-src, respectively. The immunoprecipitates bound to protein A-Sepharose beads were incubated at 37°C with 0.25 mmol/L peptide in 150 mmol/L Tris-Cl (pH 7.4), 0.16% 2-mercaptoethanol. Reactions were performed for 10 to 15 minutes, were terminated by the addition of malachite green solution (Upstate Biotechnology), and were incubated for 15 minutes before the measurement of absorbance at 630 nm. Time dependency was determined to ascertain that times of incubation fell within the linear range of the time course.

Analysis of K562 cellular differentiation. Erythroid differentiation was induced by adjusting logarithmetically growing cells to 36 μmol/L cytosine arabinoside (AraC). After 5 days, the cells were stained with benzidine solution as described.[38] Megakaryocytic differentiation was induced with 0.17 μmol/L tetradecanoyl phorbol acetate (TPA) and assessed by cellular morphology, cytochemical staining, cellular adherence, Western blot analysis with α_2 integrin antibody (Chemicon, Temecula, CA), and flow cytometry analysis with FITC-labeled CD41b ($\alpha_{IIb}\beta_3$) and CD49b (α_2 integrin) antibodies (Pharmingen).

RESULTS

Identification of PTP cDNAs expressed in hematopoietic stem cells. To identify PTPs expressed in murine hematopoietic stem cells, we used PCR amplification with degenerate oligonucleotides derived from conserved sequences in the tyrosine phosphatase domains. Template cDNA was prepared from AA4.1$^+$, Lin$^{-/lo}$, c-Kit$^+$, Sca-1$^+$ cells isolated from midgestation (day-14) fetal liver. This subpopulation is highly enriched in multipotent stem/progenitor activities.[3] Analysis of 1,300 PCR-amplified cDNA fragments showed 13 different sequences that contained the hallmark residues found in all PTPs (Table 1). Database comparison showed that 8 of the isolated PTP fragments were identical to the murine cDNAs for LRP,[39] HPTPβ,[40] CD45,[41] SHP1,[25] PTPT-9,[42] PTP 1B,[43] MEG2,[44] and PTP-BAS.[45] Two other sequences showed high homology at the amino acid level (88% and 91%) to the PTP domains of human HePTP[46] and DEP-1,[47] respectively, and therefore are likely to be their murine homologues. Similarly, two clones, PTPε-1 and PTPε-2, are likely to be related to PTPε because they are 85% and 86% homologous to the first phosphatase domain of human PTPε.[40] One of the identified PTPs corresponded to a novel sequence, and was named FLP1.

The expression patterns of all the PTP cDNAs isolated

Table 1. Type and Frequency of PTP cDNA Sequences Isolated From Mouse Cell Populations Enriched for Hematopoietic Stem Cells

Type	Frequency (%)*
Transmembrane	
LRP	21
PTPε-1	19
HPTPβ	18
CD45	12
PTPε-2	3
Intracellular	
HCP	11
PTPT-9	5
PTP 1B	4
MEG2	2
PTP-BAS	<1
DEP-1	<1
HePTP	<1
Unknown	
FLP1	<1

PTP cDNA sequences were PCR-amplified using degenerate oligonucleotides specific for the conserved sequences of PTP catalytic domains. The DNA template was cDNA from AA4.1$^+$, Lin$^{-/lo}$, Sca-1$^+$, c-Kit$^+$ fetal liver cell populations.

* Percentage numbers are based on the relative abundance of each PTP sequence calculated by differential hybridization screening of a library of 1,300 individual PCR-derived colonies.

from fetal liver stem cells were analyzed in different hematopoietic populations. Our stem cell enrichment procedure delineates four cell subpopulations: AA4.1$^+$; AA4.1$^-$; AA4.1$^+$, Lin$^{-/lo}$, c-Kit$^+$, Sca-1$^+$; and AA4.1$^+$, Lin$^{-/lo}$, c-Kit$^+$, Sca-1$^-$. As previously described,[3] the AA4.1$^+$, Lin$^{-/lo}$, c-Kit$^+$, Sca-1$^+$ subpopulation contains the bulk of long-term reconstituting hematopoietic stem cells and multipotent progenitors. Progenitor cells are also found in the AA4.1$^+$ and AA4.1$^-$, Lin$^{-/lo}$, c-Kit$^+$, Sca-1$^-$ subpopulations. More mature progenitors are present in the AA4.1$^-$ subpopulation. Two other sources of hematopoietic stem cells are adult bone marrow and day-9 yolk sac. Bone marrow hematopoietic stem cells are defined as Lin$^{-/lo}$, c-Kit$^+$, Sca-1$^+$. As for fetal liver, the Lin$^{-/lo}$, c-Kit$^+$, Sca-1$^-$ subpopulation contains more mature progenitors.[29] In day-9 yolk sac, the hematopoietic stem cells and multipotential progenitors were enriched in a cell population defined as AA4.1$^+$, WGA^{lo}, as previously described.[28] Because of the small amounts of RNA obtained from these cell subpopulations, the expression of all the different PTPs was estimated by reverse transcriptase-PCR (RT-PCR) followed by Southern blot hybridization using the PCR-derived cDNA fragments as probes. Figure 1 shows representative examples of patterns of expression for some of the PTPs. The RT-PCR analysis showed that DEP-1 is expressed in stem cell subpopulations from bone marrow, but is barely detectable in yolk sac and fetal liver stem cell subpopulations (Fig 1). Two PTPs, PTPT-9 (Fig 1) and PTP-BAS, were found to be expressed in the stem cell subpopulations from fetal liver and bone marrow but not from yolk sac. The remaining PTPs were detected in stem cell subpopulations from all three tissue sources. Moreover, PTPβ, MEG2, and FLP1 showed differential patterns of expression. As shown in Fig 1, PTPβ mRNA is present in all cell fractions analyzed, but its relative levels appear to be much higher in the fetal liver stem cell subpopulation (AA4.1$^+$, Lin$^{-/lo}$, c-Kit$^+$, Sca-1$^+$) than in the AA4.1$^+$, Lin$^{-/lo}$, c-Kit$^+$, Sca-1$^-$, which lacks long-term hematopoietic stem cells. MEG2 and FLP1 were found in all

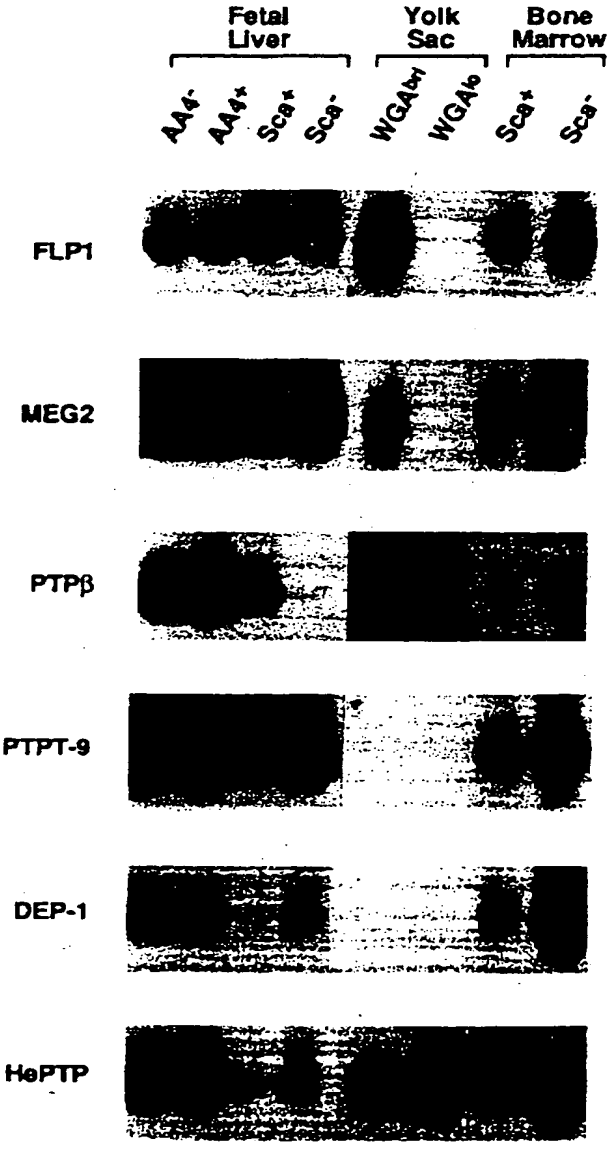


Fig 1. Expression of PTPs in fetal liver, yolk sac, and bone marrow subpopulations. Preparation of subpopulations was as described in the Materials and Methods. Expression was analyzed by RT-PCR followed by Southern blot hybridization with cDNA fragments specific for each PTP. Template cDNAs are indicated above each lane.

cell subpopulations isolated from fetal liver and bone marrow (Fig 1). However, in yolk sac, MEG2 and FLP1 cDNAs are detected in AA4.1$^+$, WGA^{hi}, which contains stem/progenitor cells but not in AA4.1$^+$, WGA^{lo}, which is lacking in stem cells and primitive progenitor cells.

The predicted amino-acid sequence of FLP1 cDNA. The expression of FLP1 in hematopoietic stem cell subpopulations from both fetal and adult sources and its cell-lineage specificity in yolk sac prompted us to further analyze this PTP. To determine the primary structure of FLP1, a mouse cDNA library (constructed from day-14 fetal liver AA4.1$^+$ cells) was screened with the PCR fragment corresponding to the FLP1 catalytic domain. Because cDNA clones initially isolated from this library did not contain the complete coding sequence, overlapping cDNA fragments corresponding to the 5′-end of the cDNA were isolated by PCR. To this purpose, oligonucleotides corresponding to FLP1 nucleotide positions 414 to 440 and 504 to 528 were used as gene specific primers to extend the cloned cDNA in the 5′ direction, using as templates cDNAs from kidney and fetal liver AA4.1$^+$ cells. The intact sequence of FLP1 (GenBank accession no. U52523) is shown in Fig 2. After sequencing several independently amplified cDNA fragments, we identified two distinct cDNAs, FLP1A and FLP1B, that differed by the presence (FLP1A) or absence (FLP1B) of a sequence in the open reading frame encoding a 24-amino acid stretch after residue at position 8 (Fig 2). Although the sequence surrounding the putative initiator codon at nucleotide positions 64-66 does not conform well to the Kozak consensus sequence, there is a purine at position −3 that is an important requirement for an initiation site.[48] The predicted sizes for the proteins encoded by the FLP1A and FLP1B cDNAs are 452 and 428 amino acids, respectively. Analysis of both sequences indicated the presence of a PTP domain located near the amino terminus, from residues 48 to 292, followed by a 160 amino acid-long carboxy terminal tail. The 66-bp 5′-untranslated region contains an in-frame stop codon at positions 13-15 and the 144-bp 3′-untranslated region contains a consensus polyadenylation signal. Comparison of the FLP1 cDNAs with those available in the database showed an almost identical sequence (GenBank accession no. U49853) that was recently identified by another group from hematopoietic progenitor cells. This cDNA is 99.8% identical at the nucleotide level to our FLP1A cDNA, and encodes for a 453 amino acid protein that is 98% identical to the FLP1A protein. Differences in both sequences include an amino acid change in position 11 and a stretch of amino acids (positions 362-367 of the FLP1 sequence and 362-368 of the hematopoietic progenitor sequence) that are different in both cDNAs. At the present time, the authenticity and/or significance of these differences are unknown.

Amino-acid sequence comparison of the catalytic domain with other PTPs showed that the closest relationships are to PTP-PEST (also named P19 or PTP G1; 51%) and PEP (47%). PTP-PEST and PEP are intracellular PTPs characterized by the presence of one catalytic domain at the amino-terminus linked to a large carboxy-terminal region that is rich in PEST sequences.[25,49-52] The amino acid sequence alignment of FLP1, PTP-PEST, and PEP with the structurally unrelated HCP and CD45 (Fig 3) shows that FLP1, PTP-PEST, and PEP constitute a separate subfamily of PTPs. PEP has recently been described to localize in the nucleus.[53] A carboxy-terminal sequence of 12 amino acids has been shown both necessary and sufficient for nuclear localization. A very similar sequence is found at the extreme carboxy terminus of PTP-PEST. Comparison of the noncatalytic sequences of FLP1 with sequences in the data base showed no significant similarities except in the last 22 carboxy-terminal residues (underlined in Fig 2), which are 68% identical to the carboxy-terminal sequence of PTP-PEST. However, FLP1 does not have subdomains containing PEST sequences found in PTP-PEST and PEP. The functions of PTP-PEST and PEP are unknown, but a recent report described that the phosphatase activity of PTP-PEST can be regulated by phosphorylation on Ser 39.[54] Interestingly, this Ser residue is conserved in the same location in both PEP and FLP1 sequences (Fig 3).

Expression of FLP1 in mouse embryos, adult tissues, and cell lines. The expression of FLP1 in tissues of adult mice was examined by Northern blot analysis. As shown in Fig 4A, a major transcript of 1.7 kb is readily detected in bone marrow, kidney, spleen, and thymus. This restricted distribution is reminiscent of HCP, which is expressed in hematopoietic tissues, kidney, and lung.[25] To examine the levels of FLP1 mRNA in different hematopoietic lineages, lymphoid and myeloid cell lines were analyzed (Fig 4B). FLP1 expression is relatively high in the thymoma line AKR1, the pro-B-cell line Ba/F3, the pre-B-cell line 70Z/3, the B-cell lymphoma WEHI-279, the leukemia line M2.4, and the macrophage lines RAW309 and WEHI-3. In contrast, FLP1 is expressed at very low levels in the leukemia line D2N, the myeloblast line M1, and the mastocytoma P815 and is virtually undetectable in NIH 3T3 fibroblasts. In addition to the 1.7-kb mRNA, a smaller FLP1 mRNA species of 1.6 kb is present in the 70Z/3 and M1 cell lines.

To examine the spatial distribution of FLP1 mRNA in embryonic tissues, we performed in situ hybridization on sections from 15.5-day-old (E15.5) and 17.5-day-old (E17.5) mouse embryos. As shown in Fig 5A, FLP1 mRNA is readily detected in E15.5 embryonic thymus and nasal epithelium. Much weaker signals are observed in kidney, liver, and intestine. Analysis of E17.5 embryos showed that FLP1 expression continues at high levels in the thymus (Fig 5B). Close examination of the in situ hybridizations shows that in both E15.5 and E17.5 thymus the FLP1 transcript is present at highest levels in cortical thymocytes (Fig 5C and D).

Subcellular localization of FLP1 protein. To identify the FLP1 protein in vivo, we generated polyclonal antibodies against a bacterial GST fusion protein containing residues 183 to 405 of the predicted FLP1 gene product. A rabbit antiserum elicited against this fusion protein efficiently immunoprecipitates the FLP1 polypeptide when transiently expressed in COS cells (data not shown). Moreover, this antibody specifically recognizes a single 48-kD protein in lysates of pro-B Ba/F3 and pre-B 70Z/3 cells (Fig 6A), which is equivalent with the molecular weight predicted from the

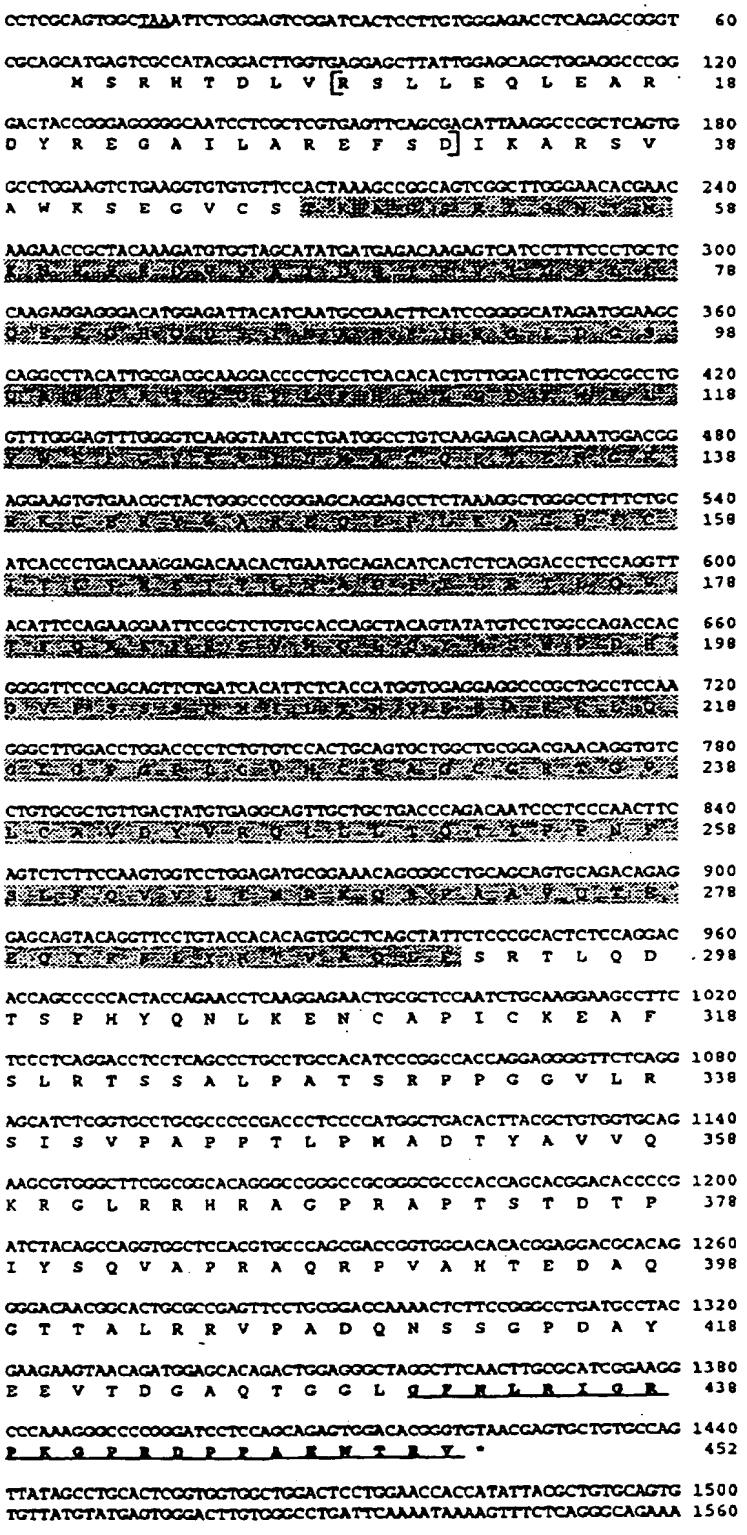


Fig 2. Nucleotide and predicted amino acid sequence of FLP1. Both nucleotide and amino acid residues are numbered at the end of each line. The deduced amino acid sequence is indicated by the single-letter code. An in-frame stop codon upstream of the ATG initiation codon is underlined. The 24 amino acids found in the FLP1A sequence but not in the FLP1B sequence are indicated with brackets. The conserved PTP catalytic domain is shaded and the potential nuclear localization signal is in bold and underlined.

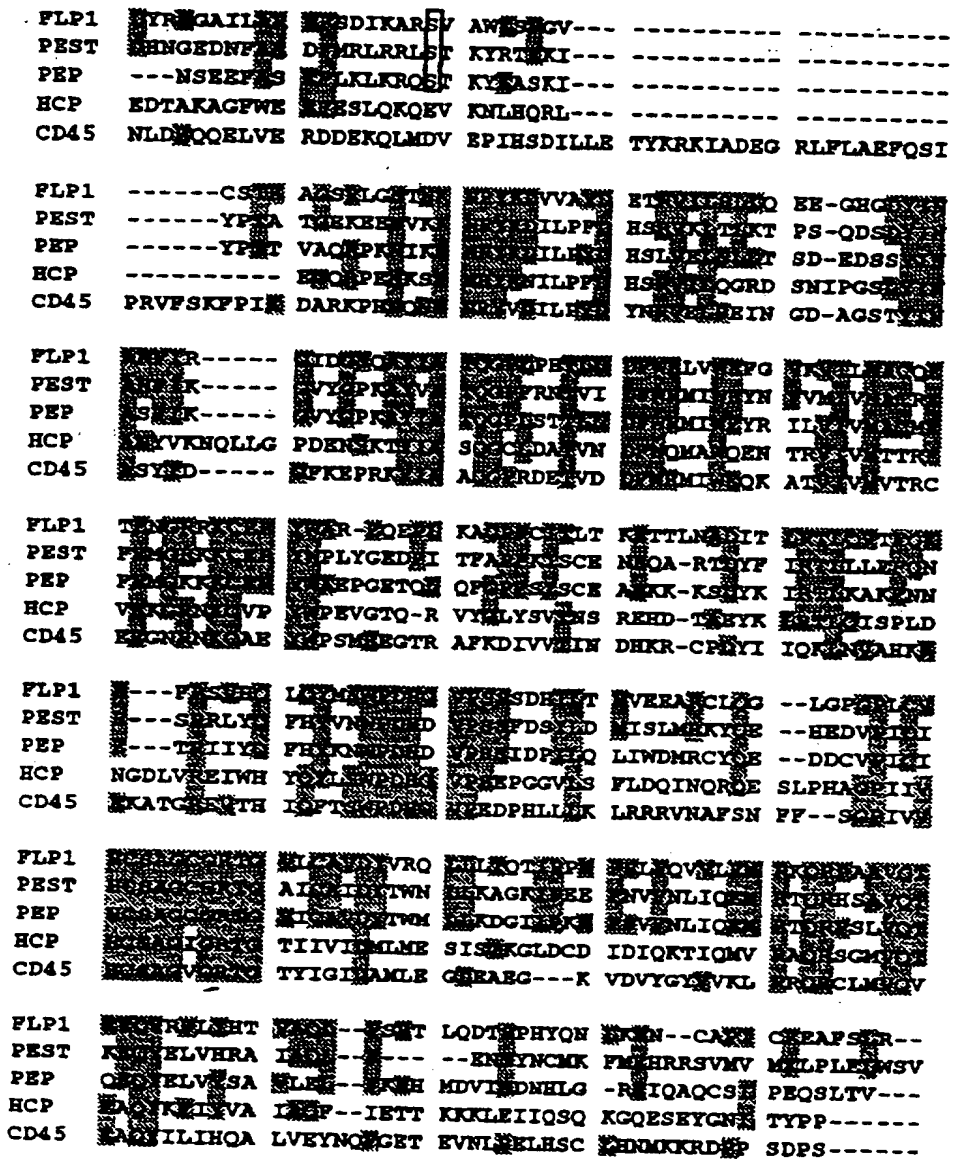


Fig 3. Similarity of FLP1 to other PTPs. Alignment of the amino acid sequences of the catalytic domains of FLP1, PTP-PEST, PEP, HCP, and CD45. Identities between FLP1 and the other sequences are shaded. Gaps introduced for optimal alignment are indicated by hyphens. The serine residue identified as an inhibitory phosphorylation site in PTP-PEST and serine residues conserved in similar locations in FLP1 and PEP are boxed.

deduced FLP1A amino acid sequence. The FLP1 protein displays the same electrophoretic mobility in COS cells and in four mouse hematopoietic cell lines, the T-cell line EL4, the myeloblast line M1 (Fig 6B), the mastocytoma line P815, and the macrophage line RAW309 (data not shown). However, in the human leukemia line K562, the FLP1 polypeptide shows a slightly smaller (45 kD) molecular weight, suggesting that, in these cells, the protein exists either as a different-size isoform or undergoes distinct posttranslational modifications.

Because the FLP1 sequence contains a putative nuclear localization signal similar to those present in PTP-PEST and PEP, we examined the subcellular localization of the FLP1 protein by immunoprecipitation and Western blot analysis. As a control, the nuclear and cytoplasmic fractions were incubated with antibodies against α-tubulin, a cytoplasmic protein. As shown in Fig 6C, the FLP1 protein is localized mostly in the nucleus of Ba/F3 cells.

Overexpression of wild-type and mutant FLP1 proteins in K562 cells. To study the function of FLP1, we investigated the effects of ectopic expression of functional or nonfunctional FLP1 protein in hematopoietic cells. As a first differentiation model, we used K562 cells. These cells have the potential to differentiate in vitro along two cell lineages.[55,56]

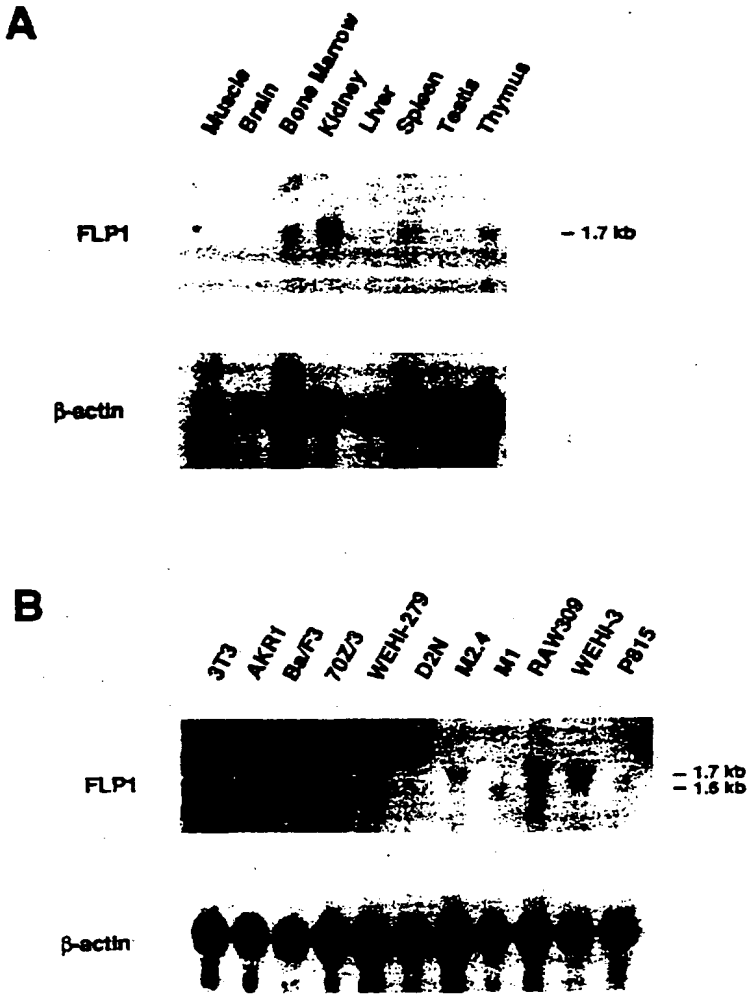


Fig 4. Expression of FLP1 in adult mouse tissues and cell lines. Total RNAs (20 μg) isolated from the indicated tissues (A) and cell lines (B) were electrophoresed in a 1.2% agarose-formaldehyde gel, transferred onto nylon membranes, and hybridized to a FLP1 cDNA probe. The sizes of the FLP1 transcripts are indicated by arrows. A mouse β-actin probe was used as a control in the same blots (lower panels).

Treatment of K562 cells with hemin or AraC can induce erythroid differentiation. In contrast, treatment with TPA leads to a macrophage-like morphology while inducing the expression of proteins associated with megakaryocytes. We first determined the pattern of FLP1 expression during differentiation of K562 cells by immunoblotlot analysis on cellular extracts nontreated and treated with AraC or TPA. As shown in Fig 7, the FLP1 protein is readily detectable in untreated cells and continues to be expressed during erythroid and myeloid differentiation. A slight increase in FLP1 levels is observed after 7 days of treatment with TPA.

We generated K562 clones overexpressing either the FLP1 protein (FLP1-A) or nonfunctional mutants of FLP1 derived by site-directed mutagenesis of the active Cys residue to a Ser residue (FLP1A-CS and FLP1-B-CS; Fig 8A). Retroviral expression vectors were constructed and electroporated with pGKneo into K562 cells. Individual clones were examined for protein expression, and four clones expressing high levels of FLP1A protein, four clones expressing high levels of FLP1A-CS protein, and four clones expressing high levels of FLP1B-CS protein (Fig 8B) were selected for further studies.

The PTP activity of the exogenous and endogenous proteins in the K562 transfectants was investigated by in vitro PTP assay. FLP1A, FLP1A-CS, or FLP1B-CS were coimmunoprecipitated with endogenous FLP1 and incubated with a synthetic phosphotyrosine peptide containing the C-terminal phosphorylation site of c-src. Dephosphorylation was allowed to proceed and the extent of PTP activity was determined using a malachite green colorimetric assay. As shown in Fig 8C, immunoprecipitates from the K562-FLP1A transfectants show a fourfold to fivefold increase in PTP activity relative to parental K562 cells. In contrast, the PTP activity in immunoprecipitates from K562 containing the mutants FLP1A-CS or FLP1B-CS is equivalent to the background levels observed for the parental K562 line. These results indicate that the K562-FLP1A clones express functional FLP1 protein and that, as previously reported for other

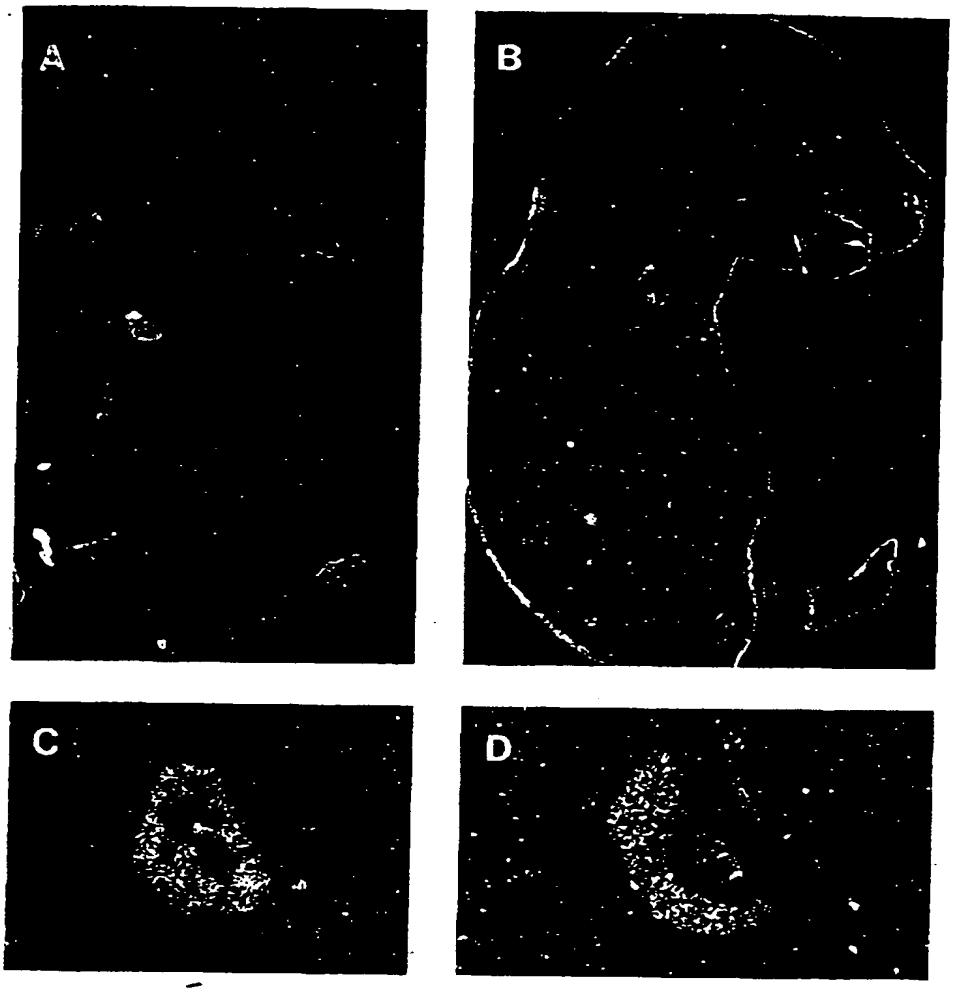


Fig 5. Expression of FLP1 in mouse embryos. In situ hybridization of saggital sections from E15.5 (A) and E17.5 (B) embryos. The signals observed in the rostral area and periphery of the embryo on (B) are not reproducible and are likely to be the result of nonspecific hybridization. (C and D) High-power magnification of the thymus from sections shown in (A) and (B), respectively.

PTPs, a single amino acid substitution (Cys to Ser in position 229) abolishes enzymatic activity. The isolation of these transfectants together with the analysis of their growth rates and saturation densities (data not shown) indicated that overexpression of functional or nonfunctional FLP1 does not affect viability or proliferation of K562 cells. Similarly, we have observed that overexpression of FLP1A and FLP1B-CS does not affect the viability or growth-factor–mediated responses of Ba/F3 cells (data not shown).

Analysis of erythroid and megakaryocytic differentiation in K562-FLP1A, K562-FLP1A-CS, and K562-FLP1B-CS cell lines. The ability of K562 cells overexpressing FLP1A and mutant FLP1A-CS or FLP1B-CS to differentiate along the erythroid lineage was assessed by treatment with AraC, a potent cell division inhibitor. The percentage of benzidine-positive cells was determined 5 days after induction. Our results indicated that elevated expression of functional or nonfunctional FLP1 does not affect the potential of K562 cells to undergo erythroid differentiation (data not shown).

Treatment of K562 cells with TPA increases cell-cell and cell-substrate adhesion and induces the expression of membrane proteins associated with the megakaryocyte lineage. To investigate whether K562-FLP1A, K562-FLP1A-CS, and K562-FLP1B-CS clones are able to differentiate along this pathway, we analyzed morphologic, cytochemical, and cell adhesion changes after TPA treatment. The addition of TPA to parental K562 and K562-FLP1A cells caused an immediate growth arrest and differentiation to morphologically recognizable megakaryocytes, with approximately 50% to 60% of the cells being adherent, over the course of 5 to 7 days (Fig 9). Cells from all the clones overexpressing the mutants FLP1A-CS and FLP1B-CS also undergo growth arrest and initial changes consistent with megakaryocyte morphology, but, in contrast to parental and wild-type FLP1A-transfected K562 cells, they contain considerably lower percentages (5% to 10%) of adherent cells. Moreover, they formed large cellular aggregates after TPA treatment. These results suggest that the expression of a catallytically inactive FLP1 protein

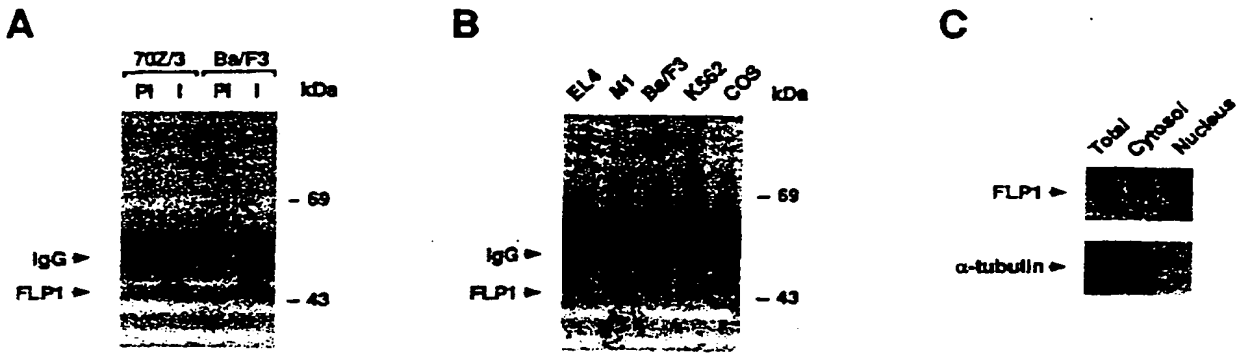


Fig 6. Identification and subcellular localization of the FLP1 protein. (A) Identification of the FLP1 protein in 70Z/3 and Ba/F3 cells. Cells were lysed in RIPA buffer and incubated with anti-FLP1 antisera. The resulting immunoprecipitates were analyzed by 8% linear SDS-PAGE, transferred to nitrocellulose, and blotted with anti-FLP1 antibody. Filters were incubated with ^{125}I-protein A and exposed to X-Omat films. The migration of FLP1 and IgG proteins are indicated by arrowheads. Coelectrophoresed molecular weight markers are indicated on the right. (B) Expression of FLP1 protein in cell lines. Lysates from the indicated cell lines were harvested and the presence of the FLP1 protein was analyzed as described in (A). (C) Subcellular localization of FLP1 in Ba/F3 cells. Cells were fractionated as indicated in the Materials and Methods. Cellular lysates of nuclear and cytosolic fractions were quantitated, normalized, resolved by 8% SDS-PAGE, and immunoblotted with antibodies to FLP1 and α-tubulin.

negatively affects the adhesion properties of K562 cells differentiated along the megakaryocytic pathway. To investigate this further, we analyzed the modulation of the major platelet integrin $\alpha_{IIb}\beta_3$, a protein complex considered an early marker of megakaryocytic differentiation, whose expression in K562 cells is markedly increased between days 1 and 3 of exposure to phorbol ester.[57] Figure 10A shows the results of flow cytometry analysis of $\alpha_{IIb}\beta_3$ expression before and after 3 days of TPA treatment. Cell-surface expression of $\alpha_{IIb}\beta_3$ increased 15- to 30-fold in parental K562 cells and in all transfectant clones, indicating that induction of $\alpha_{IIb}\beta_3$ is not affected by overexpression of wild-type or mutant FLP1. We next analyzed the expression of α_2 integrin. The $\alpha_2\beta_1$ integrin complex serves as a cell surface receptor for collagen on platelets and other cells. Elevated expression of $\alpha_2\beta_1$ accompanies megakaryocytic differentiation. In K562 cells, the induction of α_2 integrin expression, which occurs between 3 and 5 days of exposure to phorbol dibutyrate, seems to be implicated in the final steps of megakaryocyte differentiation.[57] Flow cytometry analysis showed

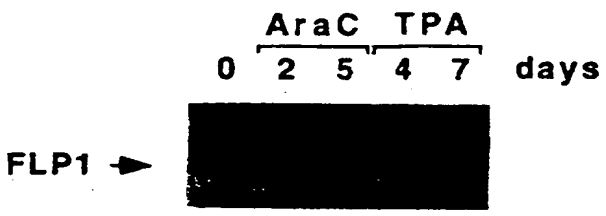


Fig 7. Western blot analysis of protein extracts before and after treatment of K562 cells with AraC and TPA. Cellular extracts were prepared and expression of FLP1 protein analyzed as described in Fig 6. The time of treatment with the inducers and the position of the FLP1 protein are indicated.

that the levels of α_2 integrin in untreated cells are lower in K562-FLP1A-CS and K562-FLP1B-CS clones than in parental K562 and K562-FLP1A cells. Furthermore, the induction of α_2 integrin expression after 4 days of TPA treatment is remarkably higher in the parental line K562 and all K562-FLP1A clones than in the K562-FLP1A-CS and K562-FLP1B-CS clones (Fig 10B). Western blot analysis on protein lysates from K562, K562-FLP1A, and K562-FLP1B-CS also showed the decreased ability of cells expressing a catallytically inactive FLP1 protein to induce α_2 integrin expression (Fig 10C).

DISCUSSION

The above studies were aimed at identifying the spectrum of PTPs that are expressed in hematopoietic stem cells. A comparable approach was previously successful in our laboratory for the isolation of PTKs from hematopoietic stem cells.[30-32] Twelve known PTP sequences and one novel PTP sequence were identified. Based on previous studies, it was anticipated that cDNA clones for hematopoietic-specific PTPs such as CD45, HCP, or HePTP would be obtained. Other expected cDNAs were PTPs widely expressed or known to be present in hematopoietic cells. These included LRP, PTPε, PTP1B, DEP-1, MEG2, PTPT-9, and PTP-BAS. However, it was not expected that the murine homolog of HPTPβ would be present at high frequency in our amplified populations. HPTPβ is a receptor-like PTP that contains an array of fibronectin type III repeats in the extracellular domain. The gene encoding HPTPβ was originally cloned from human placenta[40] and mouse brain,[58] and its pattern of expression in other tissues has not been reported. Interestingly, when we examined the distribution of mouse PTPβ in different hematopoietic populations, we found that this PTP is expressed in a fetal liver population highly enriched for stem cells but is hardly detectable in a population that lacks

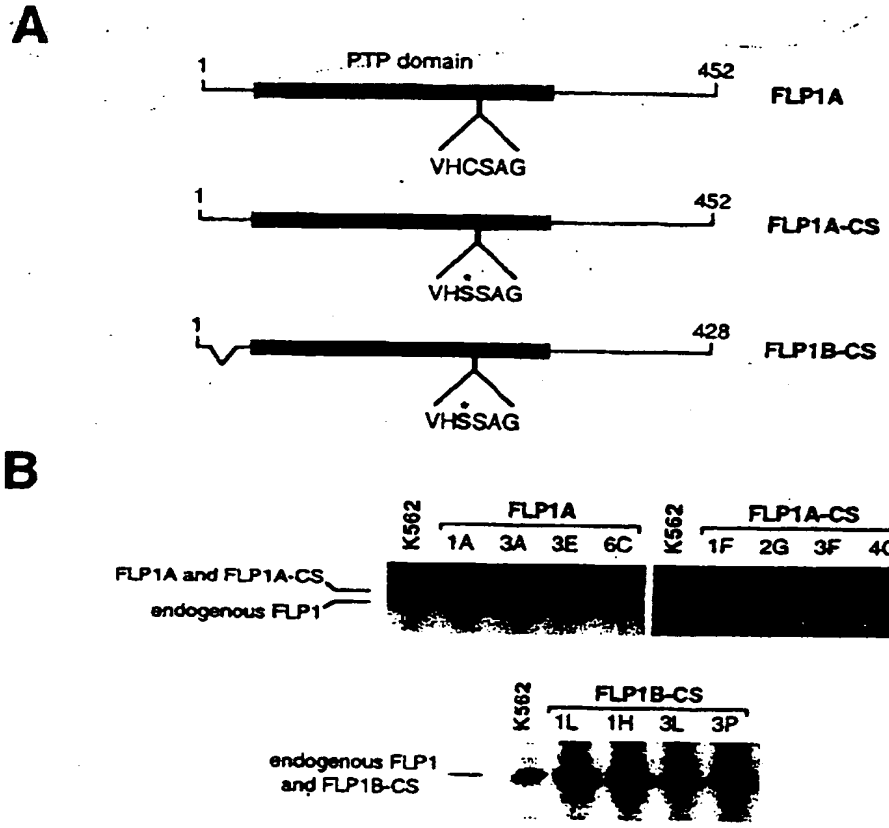
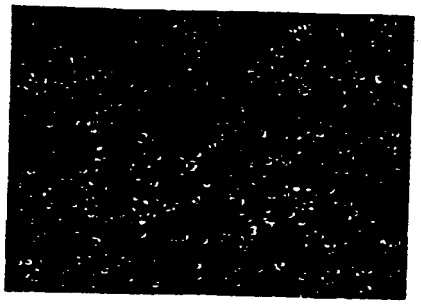
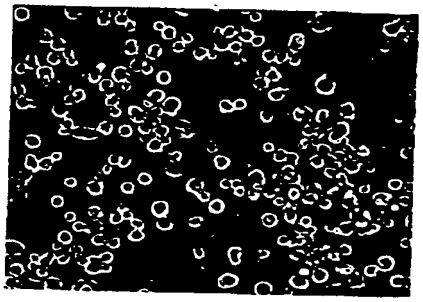


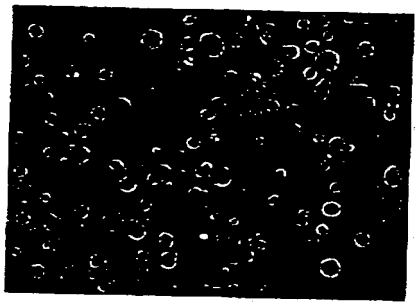
Fig 8. Generation of K562 cells stably expressing functional and nonfunctional FLP1 proteins. (A) Schematic representation of the proteins corresponding to intact FLP1 (FLP1A) and nonfunctional mutants of FLP1 (FLP1A-CS and FLP1B-CS). (B) Expression of FLP1A, FLP1A-CS, and FLP1B-CS proteins in independent K562 transfectant clones. Parental K562 and cells stably transfected with pBabe-FLP1A, pBabeFLP1A-CS, and pBabeFLP1B-CS were lysed. Protein lysates were quantitated and FLP1 proteins were immunoprecipitated and analyzed by immunoblot with anti-FLP1 antibodies. (C) PTP activity of FLP1 protein in transfectant K562 cells. FLP1 immunoprecipitates from parental K562, K562-FLP1A, K562-FLP1A-CS, and K562-FLP1B-CS were incubated with a synthetic phosphotyrosine peptide corresponding to the C-terminal domain of c-src. The relative amount of released phosphate was determined by the absorbance at 630 nm after adding a malachite green solution to the immunoprecipitates. Results represent averages of two independent experiments.



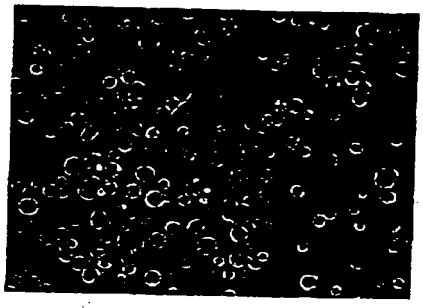
K562 (-)



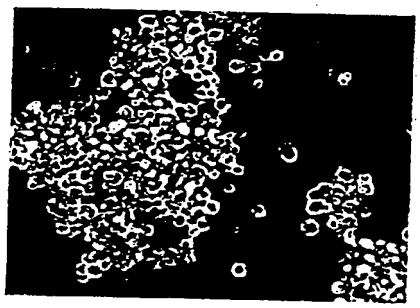
K562 (+)



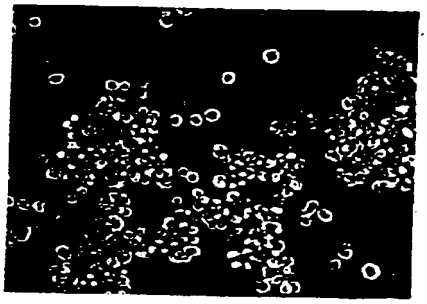
FLP1A-3A



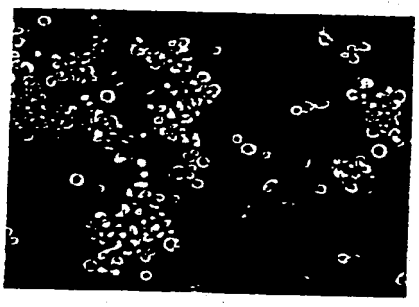
FLP1A-6C



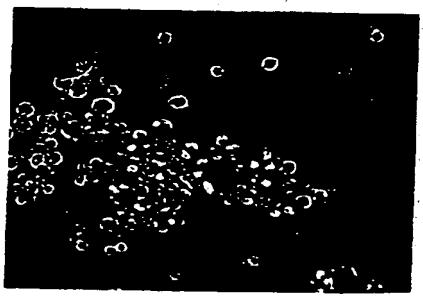
FLP1A-CS-2G



FLP1A-CS-4G



FLP1B-CS-1H



FLP1B-CS-3P

Fig 9. Morphology of K562, K562-FLP1A, K562-FLP1A-CS, and K562-FLP1B-CS cells treated with TPA. Exponentially growing cells were adjusted to 0.17 μmol/L TPA for 7 days and then photographed (original magnification × 100). −, untreated K562 cells; +, TPA-treated K562 cells. All other panels are K562-FLP1A, K562-FLP1A-CS, and K562-FLP1B-CS clones treated with TPA.

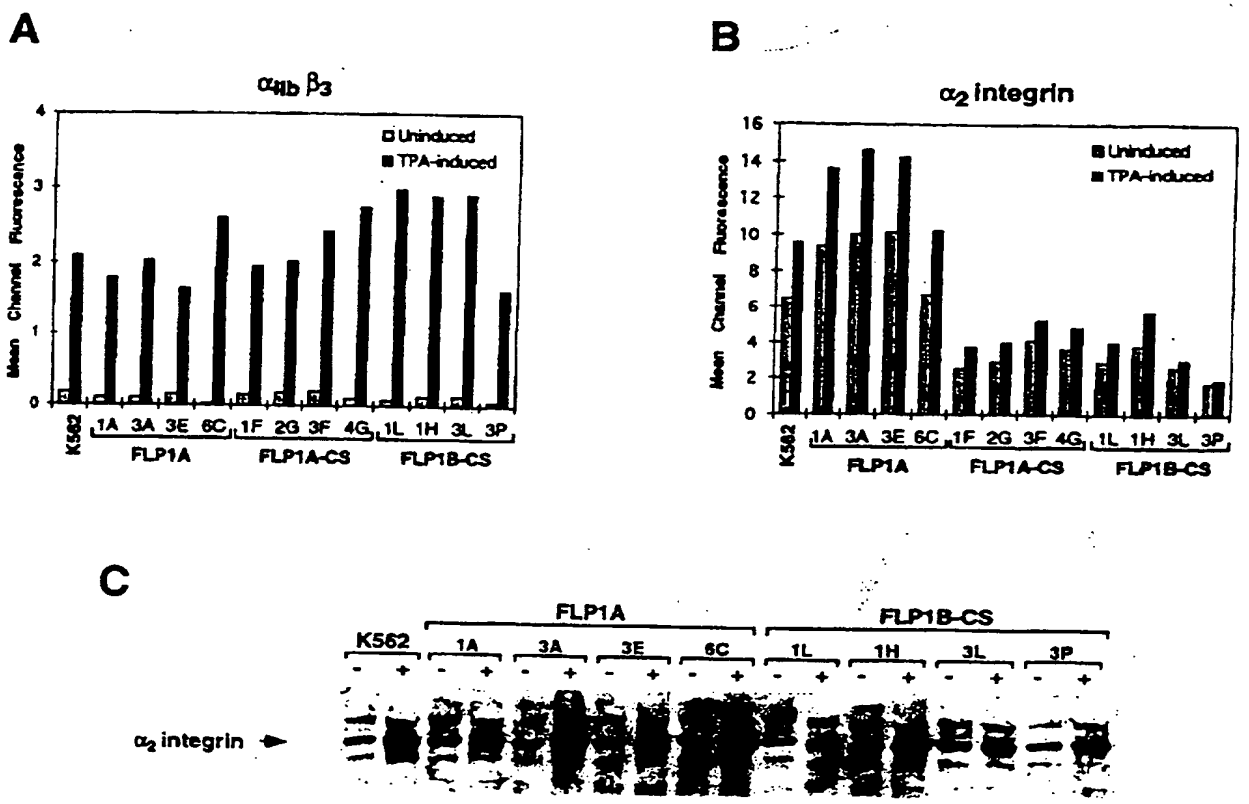


Fig 10. Induction of $\alpha_{IIb}\beta_3$ and α_2 integrin expression in K562, K562-FLP1A, K562-FLP1A-CS, and K562-FLP1B-CS cells after treatment with TPA. (A) Flow cytometry analysis of $\alpha_{IIb}\beta_3$ levels in K562, K562-FLP1A, K562-FLP1A-CS, and K562-FLP1B-CS cells either nontreated or treated with TPA for 2 days. Data represent mean channel fluorescence values after subtracting autofluorescence values of nonstained cells. (B) Flow cytometry analysis of α_2 integrin expression in untreated and treated cells for 4 days. (C) Western blot analysis of α_2 integrin expression in K562, K562-FLP1A, and K562-FLP1B-CS cells with and without TPA treatment. Cells were treated with 0.17 mmol/L TPA for 4 days, and cell extracts from untreated (−) and treated (+) cells were quantitated and subjected to Western blot analysis with α_2 integrin antibody as described in the Materials and Methods.

multipotent stem cells. Given that the presence of fibronectin III motifs has been related to functions in cell adhesion[59] and contact inhibition of cell growth,[47] PTPβ may be a candidate for mediating interactions between hematopoietic stem cells and other cellular or extracellular matrix components of the fetal liver.

The remaining PTPs identified in our screen were found to be expressed in all fetal liver and bone marrow cell populations analyzed. Two PTPs, MEG2 and FLP1, were differentially expressed in yolk sac subpopulations. Both MEG2 and FLP1 mRNAs are present in the yolk sac fraction containing stem cells but not in the fraction devoid of stem cells. In this report, we describe the cloning, expression analysis, and functional characterization of FLP1. We show that FLP1 RNA is preferentially expressed in day-15.5 and day-17.5 fetal thymus and in a limited number of adult organs, including kidney and hematopoietic tissues. These results suggest that this PTP is likely to have a function regulating tyrosine phosphorylation events specific to hematopoietic cells. Some clues about such function were provided with the characterization of FLP1 as an intracellular PTP that contains a short N-terminal domain followed by one PTP domain and a C-terminal segment with a putative nuclear localization signal. Our subcellular fractionation experiments show that the endogenous FLP1 protein is localized in the nucleus of Ba/F3 cells. Two previously identified PTPs, named PEP and PTP-PEST, contain structural motifs and localization signals similar to those found in FLP1, suggesting that these three proteins constitute a separate subfamily of PTPs. PEP is primarily expressed in hematopoietic tissues[25] and is localized to the nucleus.[53] PTP-PEST is widely distributed and has been recently reported to reside in the cytoplasm.[49,50,52] The reason for the difference in the subcellular localization of these proteins, which have similar targeting motifs, is unknown, but may reflect the existence of regulatory mechanisms that control their location in a cell-type or activation-state–dependent manner. It is also interesting to note that FLP1, PTP-PEST, and PEP contain unique C-terminal domains with no significant homologies to known sequences or polypeptide motifs. Furthermore, whereas in PEST and

PEP the C-terminal noncatalytic domains are approximately 500 amino acids long and contain sequences rich in proline, glutamic acid, serine, and threonine (PEST sequences), in FLP1 the C-terminal segment is relatively short (~160 amino acids) and does not contain PEST subdomains. These differences in the C-terminal domains of FLP1, PTP-PEST, and PEP may reflect distinct regulatory mechanisms or substrate specificities. Additionally, because PEST sequences are characteristic of rapidly degraded proteins, the C-terminal domains of PTP-PEST and PEP could be implicated in conferring rapid turnover rates to the proteins. However, both PTP-PEST and PEP have been reported to have long half-lives[49,53]; therefore, the role(s) of their PEST domains remains to be established.

In addition to restriction in expression or confinement to a particular subcellular localization, the functional specificity of PTPs is likely to be controlled by regulation of the PTP enzymatic activity. A regulatory mechanism that modulates the catalytic activity of several PTPs is phosphorylation.[60,61] One such PTP is PTP-PEST, which was recently reported to be regulated by serine phosphorylation. A serine residue at position 39 has been identified as a major inhibitory phosphorylation site on PTP-PEST in vivo after protein kinase A (PKA) activation.[15] Interestingly, serine residues are conserved in similar locations in FLP1 (position 37) and PEP (position 35), although, in the case of FLP1, the serine is not part of a consensus PKA phosphorylation site. Other putative sites of phosphorylation include S47, T57, T172, S319, and T329, which have the hallmarks of protein kinase C (PKC) phosphorylation sites, and S10, T111, S194, S202, T209, T295, T373, and S413, which possess the minimum requirements for phosphorylation by casein kinase II. Further experiments are required to resolve whether FLP1 is phosphorylated in vivo.

A second regulatory mechanism of FLP1 enzymatic activity could be the existence of protein isoforms with sequence differences within or adjacent to the PTP domain. The presence of multiple protein isoforms with variable sequences within the PTP domain has been previously described for other PTPs, including LRP,[39] LAR, PTPδ, PTPσ,[62] and PTP-PEST.[50,51] The functional significance of these differences is not known. In fetal liver and kidney, we found two FLP1 cDNAs that differ in the presence or absence of a 24 amino acid insert located in the N-terminal segment of the protein. Moreover, using PCR, we have identified two other FLP1 variants in kidney with different sequences within the PTP domain (unpublished results). Interestingly, one of these variants predicts 29 additional amino acids after position 93, a location where HCP also contains additional residues in comparison to other PTPs (Fig 3). Furthermore, in most of the cell lines analyzed, the FLP1 molecular weight is ~48 kD, which is equivalent to that predicted for the FLP1A isoform, but in K562 cells the protein is ~45 kD, which is more consistent with protein sizes encoded by smaller FLP1 variants. These results indicate that FLP1 may have many different isoforms with distinct expression patterns. We are currently investigating this issue.

To study the function of FLP1, we investigated the effects of ectopic expression of functional or nonfunctional FLP1 proteins in Ba/F3 and K562 cells. Ba/F3 are immature lymphoid cells completely dependent on IL-3 for proliferation[63] and K562 are multipotent hematopoietic cells with the ability to differentiate in vitro into erythroid and myeloid lineages.[30,46] These cell lines therefore provided appropriate cellular backgrounds to analyze the role of FLP1 in proliferation, growth factor dependence, mitogenic stimulation, and ability to influence differentiation pathways. No significant effects on cell proliferation were observed in the Ba/F3 and K562 cells overexpressing wild-type or mutant FLP1. Consistent with this finding, arrested K562-FLP1A and K562-FLP1B-CS cells undergo normal cell cycle progression after mitogenic stimulation (unpublished results). Similarly, we did not detect transformed phenotypes or any alterations in the proliferative properties of NIH 3T3 cells overexpressing wild-type or mutant FLP1 protein (unpublished results). Taken together, these results suggest that FLP1 is not directly involved in growth inhibition or mitogenic activation responses.

To gain further insight in the FLP1 biologic function, we explored whether deregulated FLP1A, FLP1A-CS, or FLP1B-CS protein expression had any effects on erythroid and myeloid differentiation of K562 cells. After treatment with AraC, parental K562 and all transfectant K562-FLP1A, K562-FLP1A-CS, and K562-FLP1B-CS cells underwent growth arrest and induced hemoglobin synthesis at similar levels. This suggests that FLP1 is not directly involved in the signaling pathways that couple AraC growth inhibition with erythroid differentiation in K562 cells. Likewise, initial morphologic and cytochemical changes consistent with megakaryocytic differentiation occurred in all transfectants after treatment with TPA. Interestingly, K562 cells expressing FLP1A-CS and FLP1B-CS showed decreases in the spreading and adhesive properties as compared with parental and K562-FLP1A cells. These results suggest that the ectopic expression of a catalytically inactive FLP1 protein might be inhibiting the changes in cell adhesion that occur during K562 megakaryocytic differentiation and indicate that this is not an isoform-specific effect. In this respect, we showed that the endogenous and TPA-induced expression levels of α_2 integrin, a protein previously shown to be essential for binding of K562 cells to collagen substrates,[57] are comparatively very low in the K562-FLP1A-CS and K562-FLP1B-CS clones. One explanation for this may be that high levels of FLP1A-CS or FLP1B-CS protein inhibit cellular differentiation. However, this seems unlikely because all K562-FLP1A-CS and K562-FLP1B-CS clones retain their abilities to undergo erythroid differentiation and to induce early megakaryocytic markers, such as the protein complex $\alpha_{IIb}\beta_3$. The ability of K562-FLP1A-CS and K562-FLP1B-CS cells to undergo morphologic changes and induce early megakaryocytic markers also excludes a direct role for FLP1 in the immediate signaling pathways initiated by protein kinase C after activation with TPA. Our results are more consistent with the interpretation that FLP1 has a function in late signaling events of K562 megakaryocytic differentiation and that the expression of an inactive form of the FLP1 acts in a

dominant negative manner. Also consistent with a function in the final stages of megakaryocytic differentiation is the fact that FLP1 protein levels increase in K562 cells after 7 days of TPA treatment. Because the expression of PTP-PEST is known to be induced during differentiation of P19 cells,[50] it could be that PTPs of the FLP1 subfamily are generally implicated in late or terminal differentiation events. Alternatively, the effect of the FLP1 mutants could be due to an ability to sequester substrates and act in a dominant positive manner, as suggested for similar mutants of other PTPs.[17] Another possible explanation is that FLP1 could be involved in the regulation of specific genes, such as the α_2 integrin gene, whose expression happens to increase during megakaryocytic differentiation. In this respect, numerous studies have shown that tyrosine phosphorylation events are directly implicated in transcriptional regulation in the interferon and other cytokine-mediated signaling pathways.[64]

In summary, our data show that FLP1, a novel PTP isolated from hematopoietic stem cells, is a nuclear PTP with restricted tissue distribution. Moreover, we provide evidence that expression of a nonfunctional FLP1 protein can affect late signaling events and downmodulate the levels of α_2 integrin during K562 megakaryocytic differentiation. This has important implications for understanding the function of FLP1. Future work will lead to the identification of functionally relevant domains and protein-protein interaction motifs in the FLP1 protein and to the functional analysis of FLP1 in cellular adhesion and terminal differentiation processes of hematopoietic cells.

ACKNOWLEDGMENT

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